Treatment with imatinib prevents fibrosis in different preclinical models of systemic sclerosis and induces regression of established fibrosis

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Abstract: OBJECTIVE: Imatinib is a small-molecule tyrosine kinase inhibitor capable of selective, dual inhibition of the transforming growth factor beta and platelet-derived growth factor (PDGF) pathways. Imatinib has previously been shown to prevent the development of inflammation-driven experimental fibrosis when treatment was initiated before administration of the profibrotic stimulus. The aim of this study was to confirm the efficacy of imatinib in a murine model of systemic sclerosis (SSc) that is less driven by inflammation and to investigate whether imatinib is also effective for the treatment of established fibrosis. METHODS: The tight skin 1 (TSK-1) mouse model of SSc was used to evaluate the antifibrotic effects of imatinib in a genetic model of the later stages of SSc. In addition, the efficacy of imatinib for the treatment of preestablished fibrosis was analyzed in a modified model of bleomycin-induced dermal fibrosis in which the application of bleomycin was prolonged and the onset of treatment was late. RESULTS: Treatment with imatinib reduced dermal and hypodermal thickening in TSK-1 mice and prevented the differentiation of resting fibroblasts into myofibroblasts. In the model of preestablished dermal fibrosis, imatinib not only stopped further progression of fibrosis but also induced regression of preexisting dermal fibrosis, with a reduction in dermal thickness below pretreatment levels. CONCLUSION: These results indicate that combined inhibition of the tyrosine kinase c-Abl and PDGF receptor might be effective in the later, less inflammatory stages of SSc and for the treatment of established fibrosis. Thus, imatinib might be an interesting candidate for clinical trials in patients with longstanding disease and preexisting tissue fibrosis.

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Treatment with imatinib prevents fibrosis in different preclinical models of SSc and induces regression of established fibrosis

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Abstract

Objective: Imatinib is a small molecule inhibitor with selective, dual inhibition of TGFβ and PDGF pathways. Imatinib has previously been shown to prevent the development of inflammation driven experimental fibrosis, when initiated before the pro-fibrotic stimulus. The aim of the present study was to confirm the efficacy of imatinib in a less inflammation driven murine model of systemic sclerosis (SSc) and to investigate, whether imatinib is also effective for the treatment of established fibrosis.

Methods: The Tight skin-1 (tsk-1) mouse model of SSc was used to evaluate the anti-fibrotic effects of imatinib in a genetic model of later stages of SSc. In addition, the efficacy of imatinib for the treatment of pre-established fibrosis was analyzed in a modified model of bleomycin induced dermal fibrosis with prolonged application of bleomycin and late onset of treatment.

Results: Treatment with imatinib reduced the dermal and hypodermal thickening in tsk-1 mice and prevented the differentiation of resting fibroblasts into myofibroblasts. In the model of pre-established dermal fibrosis, imatinib did not only stop further progression of fibrosis, but induced regression of pre-existing dermal fibrosis with reduction of the dermal thickness below pre-treatment levels.

Conclusion: These results indicate that the combined inhibition of c-abl and PDGFR might be effective for later, less inflammatory stages of SSc and for the treatment of established fibrosis. Thus, imatinib might an interesting candidate for clinical trials with patients with longstanding disease and pre-existing tissue fibrosis.

Introduction

Systemic sclerosis (SSc) is a chronic fibrotic disorder of unknown etiology that affects the skin and a variety of internal organs. During the course of the disease, an excessive accumulation of extracellular matrix (ECM) components develops in the skin and in involved organs (1). The resulting fibrosis disrupts the physiological structure of the affected tissues and can lead to severe dysfunction of the involved organs. Tissue fibrosis causes not only a major cause of morbidity in SSc, but contributes significantly to the increased mortality of SSc patients as well (2). The accumulation of ECM in SSc is mediated by activated fibroblasts, which produce increased amounts of extracellular matrix proteins (3). TGFβ and PDGF are considered to play important roles for fibroblast activation in SSc (3).

Imatinib mesylate (Gleevec/Glivec) is an orally administered drug, which is widely used for the treatment of bcr-abl positive chronic myelogenous leukemia and gastrointestinal stromal tumors. Previous clinical trials in patients with chronic myelogenous leukemia demonstrated that imatinib is relatively well tolerated (4). Imatinib targets specifically TGFβ and PDGF signaling pathways by inhibiting the tyrosine kinase activity of c-abl and PDGF receptors and thus interferes simultaneously with two major pathways for the activation of fibroblasts in SSc (5). Recently, we could demonstrate that imatinib prevented the development of dermal fibrosis upon challenge with bleomycin, when the treatment was initiated before the first injection of bleomycin (6). Imatinib has also been shown to be preventive in inflammatory models of other organ fibrosis (7-9).

In SSc, prominent tissue inflammation is mainly restricted to very early stages of the disease and rarely occurs in longstanding disease. Thus, data obtained from these inflammatory models of tissue fibrosis mimic early stages of SSc, but are less representative for later stages of SSc. Another limitation of previous studies is that they only analyzed, whether imatinib can prevent the development of fibrosis. However, most patients with diffuse SSc are seen by the rheumatologist, when significant tissue fibrosis has already

occurred. The therapeutic aim in these patients would thus be to stop progression of their disease and even induce regression of pre-existing fibrosis. Ideal anti-fibrotic drugs should sufficiently decrease the production of collagen to shift the balance between matrix synthesis and matrix degradation towards matrix degradation with subsequent reduction of the pre-existing accumulation of extracellular matrix. Kay et al. demonstrated recently, that imatinib induced regression of the skin score in two patients with nephrogenic systemic fibrosis (10). Considering the potent anti-fibrotic effects of imatinib on prevention of fibrosis in inflammatory models and the findings by Kay et al., we hypothesized that imatinib might also be effective in fibrosis models mimicking longstanding disease and for the treatment of pre-established tissue fibrosis.

Material and methods

Prevention of fibrosis in tight-skin 1 mice by imatinib

To confirm the efficacy of imatinib in a model of SSc that is less dependent on inflammatory changes than bleomycin induced dermal fibrosis, the anti-fibrotic potential of imatinib was evaluated in the tight-skin-1 (tsk-1) mouse model of SSc. Due to a dominant mutation of the fibrillin-1 gene, the phenotype of tsk-1 is characterized by an increased dermal and hypodermal thickness (11, 12). Tsk-1 mice were interbred with pa/pa mice, in which a recessive mutation (pa) induces a light grey color of the fur and pink eyes. As the fibrillin gene is genetically linked to the pa gene, mice can be pre-screened for the Tsk-1 mutation by the color of the fur and of the eyes. All mice with black fur and eyes carry the dominant tsk-1 mutation and are heterozygous for the pale mutation. In contrast, mice with light grey fur do not carry the tsk-1 mutation, but are homozygous for the mutated pale gene. Apart from the change in skin color, the pale mutation itself does not alter skin physiology or fibrogenesis. Imatinib was dissolved in 0.9 % sodium chloride (NaCl) and injected intraperitoneally in a total volume of 100 µl. Three groups with a total of 27 mice were analyzed. The injection scheme is summarized in Figure 1a. One group of tsk-1 mice was treated with imatinib at a dose of 150 mg/kg/d, another tsk-1 group was injected with the solvent NaCl. The last group consisted of pa/pa mice on the same genetic background not carrying the tsk-1 mutation ("controls"), which also received intraperitoneal injections of NaCl. The treatment was started at an age of five weeks. After five weeks of treatment, mice were sacrificed by cervical dislocation and the skin processed further for histological analysis.

Treatment of established bleomycin induced dermal fibrosis with imatinib

Skin fibrosis was induced in 6-weeks-old DBA mice by local intracutaneous injections of 100 µl of bleomycin dissolved in 0.9% NaCl at a concentration of 0.5 mg/ml every other day in defined areas of 1.5 cm^2 on the upper back. The injection schemes for the six different

groups are summarized in Figure 1b. Briefly, one group of mice was sacrificed after 3 weeks of treatment with bleomycin to analyze the fibrotic changes before treatment with imatinib. Another group of mice was sacrificed after 6 weeks of injections with bleomycin. The third group was injected for 3 weeks with bleomycin and then for next 3 weeks with NaCl to control for spontaneous regression of fibrosis. To assess the effects of imatinib on established fibrosis, mice were challenged with bleomycin for 6 weeks and treated in parallel with imatinib at doses of 150 mg/kg/d for the last 3 weeks. Two groups of mice receiving intracutaneous injections of 100 µl 0.9% NaCl for 3 weeks and for 6 weeks respectively were used as controls. All mice that were not treated with imatinib received intraperitoneal injections of NaCl. A total of 49 mice were analyzed.

Histological analysis

Skin sections were stained with hemalaun/eosin for better visualization of the tissue structure. The dermal thickness was analyzed with a Nikon Eclipse 80i microscope (Nikon, Badhoevedorp, Netherlands) by measuring the maximal distance between the epidermal-dermal junction and the dermal-subcutaneous fat junction at four different skin sections of each mouse as described (13). The hypodermal thickness was determined by measuring the thickness of the subcutaneous connective tissue beneath the panniculus carnosus at four different sites at the upper back in each mouse. The evaluation was performed by two independent examiners.

Detection of myofibroblasts

For quantification of myofibroblasts, skin sections were deparaffinized and incubated with 5% bovine serum albumin for 60 min. Cells positive for α-smooth muscle actin (αSMA) were detected by incubation with monoclonal anti-αSMA antibodies (clone 1A4, Sigma-Aldrich, Steinheim, Germany) for two hours at room temperature followed by incubation with

3% hydrogen peroxide for 10 min. Goat-anti-rabbit antibodies labeled with horseradish peroxidase were used as secondary antibodies (Dako, Hamburg, Germany). The expression of αSMA was visualized with 3,3-diaminobenzidine tetrahydrochloride (DAB) (Sigma-Aldrich). Monoclonal mouse IgG antibodies (Calbiochem, San Diego, CA, USA) were used for controls (14).

Statistics

Data are expressed as mean ± standard deviation. The Mann-Whitney-U-test was used for statistical analyses. A p-value of less than 0.05 was considered statistically significant.

Results

Imatinib corrects the tight skin-1 phenotype

We first aimed to assess whether imatinib is effective in an in-vivo model of dermal fibrosis that is largely independent from inflammation. Thus, we evaluated the efficacy of imatinib in the tsk-1 mouse model. Tsk-1 mice are characterized by a modest increase in dermal thickness and strongly increased hypodermal thickness. Treatment with imatinib almost completely prevented the histological changes in tsk-1 mice (Figures 2a-d). The dermal thickness was increased in tsk-1 mice by 40 ± 6% compared to pa/pa mice ($p = 0.003$). Treatment with imatinib reduced the dermal thickness to normal levels (- 5% ± 8%; $p = 0.005$) (Figures 2a and 2b). The increased thickness of the hypodermis in tsk-1 mice was also significantly reduced by imatinib from 298 ± 9 % to 84 ± 6 % ($p = 0.005$) (Figures 2a and 2c).

Myofibroblasts are considered as major effector cells for fibrosis. Imatinib significantly reduced the differentiation of resting fibroblasts into myofibroblasts in tsk-1 mice (Figure 2d). The number of myofibroblasts was reduced from 304 ± 28 % in untreated tsk-1 mice to normal in tsk-1 mice treated with imatinib (-10% ± 10%; $p = 0.0001$).

Of note, treatment with imatinib completely normalized the dermal thickness and myofibroblasts counts. The mean dermal thickness and the numbers of myofibroblasts did not differ between control mice and tsk-1 mice treated with imatinib ($p = 0.34$ and $p = 0.85$, respectively).

Imatinib induces regression of established fibrosis

While the prevention of fibrosis is a major aim in SSc, the clinical situation is most often characterized by patients that present with already established fibrosis. Thus, potential anti-fibrotic drugs for SSc should not only prevent fibrosis, but also induce regression of pre-existing tissue fibrosis. To evaluate the efficacy of imatinib for the treatment of established fibrosis, a modified model of bleomycin induced dermal fibrosis was used. Consistent with

previous studies, the dermal thickness increased by 50 ± 2 % after 3 weeks of injections with bleomycin ($p = 0.002$ compared to controls) (data not shown). Prolonged injections of bleomycin increased the dermal thickness further. When the challenge with bleomycin was continued for additional 3 weeks to a total of 6 weeks, the dermal thickness increased by 68 ± 3 % ($p = 0.001$ compared to 3 weeks and $p = 0.008$ compared to controls). Treatment with imatinib for the last 3 weeks of bleomycin injection did not only stop the further progression of fibrosis, but induced regression of pre-existing matrix accumulation and decreased the dermal thickness below pre-treatment levels (Figures 3a and b). The dermal thickness in mice treated with imatinib for the last 3 weeks was significantly reduced to 24 ± 4 % compared to mice injected with bleomycin for 6 weeks ($p = 0.002$). The dermal thickness in mice injected with bleomycin for 6 weeks and treated with imatinib for the last 3 weeks was also significantly lower than in mice challenged with bleomycin for 3 weeks (24 ± 4 % vs 47 ± 1 % compared to controls, $p = 0.003$). These findings suggest that imatinib does not only prevent the development of fibrosis, but can also induce regression of pre-existing fibrotic damage.

Discussion

We have previously shown that imatinib prevents the development of fibrosis in the mouse model of bleomycin induced dermal fibrosis (6). This model of dermal fibrosis is characterized by dense inflammatory infiltrates in lesional skin. Inflammatory cells are thought to contribute to the initial activation of resident fibroblasts by the release of pro-fibrotic mediators. Thus, the mouse model of bleomycin induced dermal fibrosis mimics early stages of SSc, but is less representative for later stages of SSc, where inflammatory infiltrates are scarce (3). To evaluate the anti-fibrotic efficacy of imatinib in a model for later stages of SSc, we used the tsk-1 mouse model, in which persistent overproduction of extracellular matrix proteins occurs in the absence of inflammatory infiltrates (12). We demonstrate potent anti-fibrotic effects of imatinib in tsk-1 mice with prevention of histological changes and inhibition of myofibroblast differentiation. Thus, our data obtained in tsk-1 mice indicate that imatinib might not only be effective for prevention of fibrosis in early, inflammatory stages of SSc, but also in later stages of SSc, when inflammation is not dominant any more.

Regression of established fibrosis upon treatment with imatinib is probably mediated by a relative overweight of matrix degradation relative to neo-synthesis of extracellular matrix components. In our previous studies (6; 13), we demonstrated that the combined inhibition of c-abl and PDGFR decreases the synthesis of collagen by up to 80 %. Direct effects of imatinib on matrix-degrading enzymes or their inhibitors were not observed. Thus, imatinib might induce regression of fibrosis via its potent inhibitory effects on collagen synthesis leading to a relative overweight of matrix degradation rather than by direct effects on matrix degradation.

The data obtained in tsk-1 mice in the present study and in the model of acute bleomycin induced dermal fibrosis in our previous study (6) demonstrate that imatinib prevents effectively the development of dermal fibrosis in different preclinical models of SSc. If translated into clinics, prevention of fibrosis is mainly relevant for patients with very early, progressive SSc, which are at high risk to develop significant morbidity due to the progressive

deposition of extracellular matrix proteins in different organs. In contrast, the majority of patients present to rheumatology clinics with already some extent of established fibrosis. Thus, treatment need for established fibrosis is a frequent and urgent treatment aim in this disease. In these patients treatment would remove pre-existing fibrotic changes and potentially reduce organ dysfunction. Using a modified model of bleomycin induced dermal fibrosis with prolonged application of bleomycin and late onset of treatment, we demonstrate that imatinib does not only halt progression, but even reduces pre-existing dermal fibrosis and decreases dermal thickness below pre-treatment levels despite ongoing challenge with the pro-fibrotic stimulus bleomycin. Thus, imatinib might not only stop progression, but also induce regression of tissue fibrosis. Imatinib might therefore also be an interesting candidate for clinical trials SSc with patients with extensive, longstanding disease and pre-existing fibrotic organ damage.

However, potential anti-angiogenic adverse effects of imatinib are an important concern in particular in SSc patients with severe microvascular disease and recurrent ulcers. Although imatinib might not affect endothelial cells directly (15), careful monitoring of SSc patients for exacerbation of vascular disease is warranted in clinical trials with imatinib.

Taken together, we have shown that imatinib exerts potent anti-fibrotic effects in two in vivo models of SSc with different underlying pathologic mechanisms. Imatinib was effective for prevention of fibrosis and for treatment of established dermal fibrosis. Furthermore, imatinib exerts potent anti-fibrotic effects also in preclinical models of other fibrotic diseases (7-9). The results of the present study indicate that the combined inhibition of c-abl and PDGFR might be effective for the treatment of established fibrosis as well. These findings enhance our excitement about the upcoming results of clinical trials with imatinib in SSc and in other fibrotic disorders. The results from these trials including their safety analyses must be awaited, before imatinib can be used routinely in the daily clinical care of SSc patients.

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Figure legends

Figure 1: Experimental design for the treatment of tsk-1 mice (**Figure 1a**) and bleomycin induced, established dermal fibrosis (**Figure 1b**) with imatinib. † = sacrifice

Figure 2: Anti-fibrotic effects of imatinib in tsk-1 mice. **Figure 2a**: Treatment with imatinib reduces significantly dermal and hypodermal thickening in tsk-1 mice compared to mock treated tsk-1 mice. Representative sections are shown in 40 fold magnification. Upper bar: Dermal thickness. Lower bar: Hypodermal thickness. **Figure 2b**: Reduction of the dermal thickness in tsk-1 mice by imatinib. **Figure 2c**: Decreased hypodermal thickness in tsk-1 mice treated with imatinib. **Figure 2d**: Prevention of myofibroblast differentiation by imatinib.

Figure 3: Imatinib induces regression of pre-existing skin fibrosis in a modified model of bleomycin induced fibrosis. **Figure 3a**: Treatment with imatinib prevents not only further matrix accumulation, but also induces regression of pre-existing fibrosis. Representative sections are shown in 100 fold magnification. **Figure 3b**: Treatment with imatinib decreases dermal thickness below baseline levels.